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Movement ecology and sex are linked to barn owl microbial community composition

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Abstract

The behavioural ecology of host species is likely to affect their microbial communities, because host sex, diet, physiology, and movement behaviour could all potentially influence their microbiota. We studied a wild population of barn owls (*Tyto alba*) and collected data on their microbiota, movement, diet, size, coloration, and reproduction. The composition of bacterial species differed by the sex of the host and female owls had more diverse bacterial communities than their male counterparts. The abundance of two families of bacteria, Actinomycetaceae and Lactobacillaceae, also varied between the sexes, potentially as a result of sex differences in hormones and immunological function, as has previously been found with Lactobacillaceae in the microbiota of mice. Male and female owls did not differ in the prey they brought to the nest, which suggests that dietary differences are unlikely to underlie the differences in their microbiota. The movement behaviour of the owls was associated with the host microbiota in both males and females because owls that moved further from their nest each day had more diverse bacterial communities than owls that stayed closer to their nests. This novel result suggests that the movement ecology of hosts can impact their microbiota, potentially on the basis of their differential encounters with new bacterial species as the hosts move and forage across the landscape. Overall, we found that many aspects of the microbial community are correlated with the behavioural ecology of the host and that data on the microbiota can aid in generating new hypotheses about host behaviour.

KEYWORDS

behavioural ecology, microbiome, movement ecology, sexual differentiation, *Tyto alba*

1 | INTRODUCTION

Animals and the microbes living within or on them have a symbiotic relationship characterized by a variety of important interactions. Hosts can affect the composition of their microbiota through diet (Delsuc et al., 2014; Godoy-Vitorino et al., 2011; Muegge et al., 2011), behaviour (Risely, Waite, Ujvari, Hoyer, & Klaassen, 2018; Smits et al., 2017; White et al., 2010), genes (Goodrich, Davenport, Waters, Clark, & Ley, 2016), and physiology and health (Ganz et al., 2017). Microbes may affect their hosts by causing or preventing disease (Soler, Martín-Vivaldi, Peralta-Sánchez, Arco, & Juárez-García-Pelayo, 2014; Vågene et al., 2018), aiding in digestion (Hehemann et al., 2010; Kohl, Connelly, Dearing, & Forbey, 2016; Kohl, Weiss, Cox, Dale, & Dearing, 2014), and interacting with host development (McFall-Ngai, 2014). While much of the microbiota is obtained from the surrounding environment or diet (Moeller et al., 2013, 2017; Moeller, Suzuki, Phifer-Rixey, & Nachman, 2018), bacterial colonization of the host is also influenced by the host environment, because it has been established that host species in the same geographic location often have distinct microbiota reflecting their evolutionary history (García-Amado et al., 2018; Hird, Sánchez, Carsten, & Brumfield, 2015; Kropáčková et al., 2017; Phillips et al., 2012). Some microbes are vertically inherited (Moeller et al., 2016, 2018; Ochman et al., 2010), which allows for co-evolution with their hosts and potentially large effects on the fitness of the interacting species (Brucker & Bordenstein, 2013). The complexity of the possible interactions between hosts and their microbes points to the need to identify the major ecological and environmental factors that shape the microbial communities of a host. Work on model species in the laboratory has been vital for increasing our understanding of how hosts and microbes affect one another (Goodrich et al., 2016; Markle et al., 2013; Moeller et al., 2018). However, studies of free-ranging animals in the wild are also needed if we are to understand how hosts and microbes interact in a more natural context in which environmental and ecological effects play a larger role (Hird, Carstens, Cardiff, Dittmann, & Brumfield, 2014; Hird et al., 2015; Moeller et al., 2013; Schnorr et al., 2014).

Many aspects of the behavioural ecology of wild species are likely to affect their microbiota. Males and females often have distinct behavioural ecologies (Davies, Krebs, & West, 2012), which could affect their microbiota in a variety of ways. The two sexes may have different diets (Temeles, Pan, Brennan, & Horwitt, 2000), which could affect the prevalence of particular gut microbes (Muegge et al., 2011; Schnorr et al., 2014). Males and females can have different body sizes (Corl, Davis, Kuchta, Comendant, & Sinervo, 2010) and microbial diversity has been correlated with body mass differences within and among species (Gao et al., 2018; Reese & Dunn, 2018). The sexes may also differ in social and sexual interactions, which can affect how microbes spread among individuals (Levin et al., 2016; White et al., 2010). Males and females can have different microbiota during the breeding season (Escallón, Belden, & Moore, 2019), which is a period of time when the sexes may differ profoundly in parental care, physiology, and stress levels. In addition, there are often

differences between the sexes in their immune function, which can lead to differences in their microbiota (Fransen et al., 2017; Markle et al., 2013; Yurkovetskiy et al., 2013). Thus, there is significant potential for the two sexes to have distinct microbiota, especially during periods in which males and females assume different reproductive roles.

Host movement behaviours may also influence the microbiota of hosts. The movement ecology of individual hosts can differ in many aspects including activity pattern (e.g., moving short vs. long distances), habitat preference, territory size, interaction with different individuals, and whether they choose to disperse from a residence area. All of these differences in movement ecology may impact the microbiota by affecting host contact with particular microbes. Microbial communities can vary at both large (Moeller et al., 2017) and small spatial scales (Suzuki & Nachman, 2016), so hosts that reside in different localities may be colonized by different microbes. Hosts that move through and interact with a greater diversity of environments will probably have higher microbial diversity than their more sedentary counterparts if microbial species are patchily distributed in the environment. Alternatively, movement behaviours may be indirectly linked to microbiota if host physiology impacts both movement ecology and microbial communities. For example, migrating birds may experience temporary gut atrophy, and migrant populations have been found to have lower microbial diversity than conspecific populations of resident birds (Risely et al., 2018). Although some population studies have examined the influence of migratory behaviour on host microbiota, the effects of the movement ecology of individual hosts on their microbiota have rarely, if ever, been explored. Detailed tracking data for individual hosts is required to determine whether and how an animal's movement affects its microbiota. Such information could increase our understanding of the ways in which exposure to new microbial species in the landscape affects host microbiota.

Host physiology, reproductive state, and measures of reproductive fitness may also be associated with differences in host microbiota. In humans, the physiological changes accompanying pregnancy are associated with changes in the gut microbiota (Koren et al., 2012). The reproductive periods of wild species may similarly affect their microbiota. Measures of host reproductive fitness (e.g., number of offspring) might also be associated with different microbial communities if the condition of the host influences both its fitness and its microbiota. Healthy hosts can have different microbiota than unhealthy hosts (Ganz et al., 2017) and healthy individuals can have larger clutch sizes than unhealthy individuals (Merilä & Andersson, 2016). In addition, host stress hormone levels can be associated with both reproductive fitness (Ouyang, Sharp, Dawson, Quetting, & Hau, 2011) and with changes to their microbiota (Noguera, Aira, Pérez-Losada, Domínguez, & Velandó, 2018). Thus, microbial species composition may be associated with a wide variety of traits related to the physiology and reproduction of the hosts.

We conducted a broad study of the behavioural ecology of a population of barn owls (*Tyto alba*) aimed at revealing the principle host phenotypes associated with variation in cloacal microbial

composition, focusing on sexual differences and variation in host movements. Detailed behavioural ecological studies of barn owls are possible because they can be trapped at their nest boxes, allowing for the efficient collection of a wide range of data on individual owls and their offspring. Male and female barn owls differ markedly in their behaviour during the nesting period: females lay and incubate eggs, protect the nest, and care for nestlings (e.g., feed them from prey brought by males), while males forage intensively to feed the entire family until adult females resume intensive hunting around three weeks after hatching the first nestling. We tested for differences between the microbiota of males and females, which could arise from their distinct behavioural ecologies, such as behavioural differences while nesting or physiological differences. We also assessed how host microbiota changed with the age of their nestlings, given that females change their behaviour as the nestlings grow and that females go from being fed by the male early in the nesting period to feeding themselves later in that period. In addition, we tested whether morphology (e.g., mass, wing length) and reproductive parameters (clutch size, laying date, number of fledglings, etc.) of the owls were correlated with their microbiota, as host size and health could affect both their reproduction and microbial communities. We collected detailed movement data from barn owls that were harnessed with a reverse-GPS tracking system (Weiser et al., 2016) to examine whether the microbial composition of individual owls was correlated with the size of their home range and/or with average daily flight distance from their nest. Using camera traps, we obtained dietary data on individual birds, which allowed us to test whether male and female owls differed in the species of the prey that they caught. Finally, we examined whether the microbiota varied with the birds' feather coloration, because the amount of melanin on the feathers has previously been correlated with immune function (Roulin, Jungi, Pfister, & Dijkstra, 2000; Roulin, Riols, Dijkstra, & Ducrest, 2001) and dietary differences (Charter, Peleg, Leshem, & Roulin, 2012; Roulin, 2004b) among barn owls. Overall, our aim was to gain insight into the major factors that structure the cloacal microbiota of a wild bird species to better understand how host sex, movement behaviours, and reproduction impact their microbial communities.

2 | MATERIALS AND METHODS

2.1 | Owl capture, monitoring, and data collection

We studied a population of barn owls in the Hula Valley in northern Israel (Figure 1) from 12 April–5 July in 2017 using methods approved by the ethics committee of the Hebrew University of Jerusalem (permit NS-16-14801-2) and the UC Berkeley IACUC (No. AUP-2016-04-8665-1). One of the authors (M.C.) monitored barn owl nest boxes from 1–8 times (mean = 3.7 times per nest box) to determine nest box occupancy and to track owl reproduction across the season (Figure 1b). Data were collected on: (a) clutch size; (b) age of the oldest nestling, determined by back calculations using wing

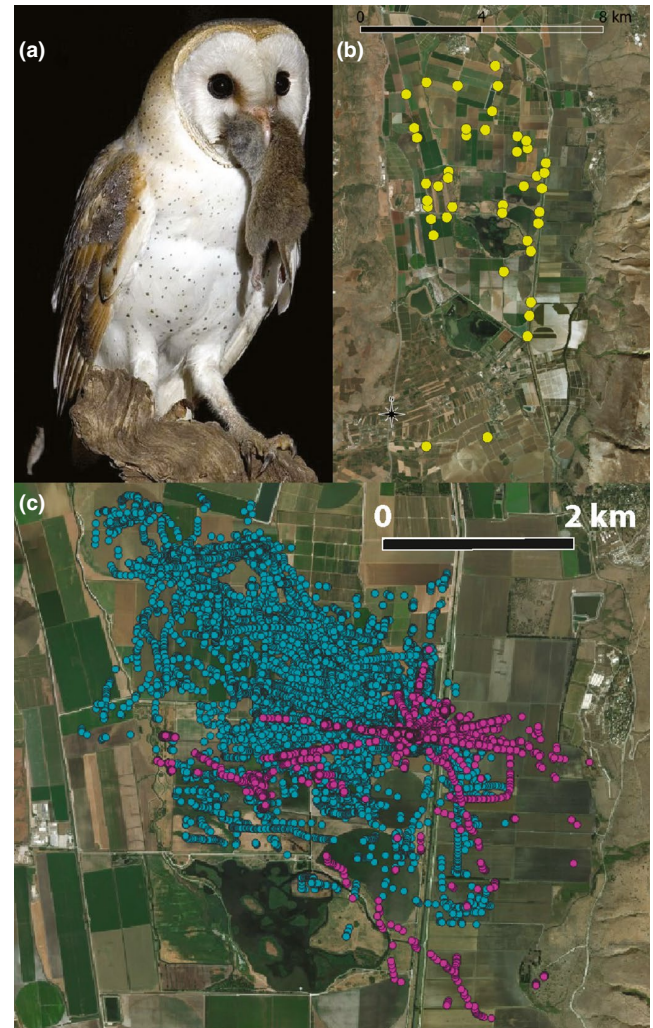


FIGURE 1 (a) A female barn owl with a prey item (Photo: Amir Ezer). Diet data was collected with camera traps at nest boxes. (b) Map of the study site in the Hula Valley, Israel. Yellow points show the position of the nest boxes of the owls that were sampled for this study. (c) An example of the movement data that was collected on the owls. The data are for a mated pair of owls collected over a consecutive 15 day period, with blue circles for the male (20,019 localizations) and purple circles for the female (70,249 localizations)

length (Roulin, 2004a); (c) laying date (the date on which the first egg was laid); (d) the number of young hatched; (e) brood size at fledging, measured by the number of nestlings when the oldest individual was 53 days old (Charter, Izhaki, & Roulin, 2018); (f) fledging success, measured by the percentage of hatchlings that survived to fledging from each brood, and (g) egg productivity, measured by the percentage of eggs that survived to fledging from each brood. We inferred laying dates by identifying the oldest nestling using wing length (the oldest nestling has the largest wings due to the asynchronous incubation of barn owls) and then back-calculating laying date from the age of the oldest nestling plus an average incubation period of 32 days (Roulin, 2004a).

Adults of both sexes were captured either during the day inside the nest box or at the entrance of the nest box at night after at least one nestling had hatched to minimize the probability of nest

abandonment. The cloaca of each adult was swabbed in the field to obtain a sample of the microbiota of each owl. The swabs were sterile Flexible Mini Tip FLOQSwabs (Copan Diagnostics Inc.) The full head of the swab was inserted into the cloaca in a circular motion and then the inside of the cloaca was swabbed in three circular motions. The swab was placed in 95% ethanol, stored at -20°C in the field for 2–4 days, and then switched to -80°C for long-term storage. Data on the mass (g), wing length (mm), and tarsus length (mm) of each adult owl was also collected after trapping. The sex of the birds was determined when they were trapped, as only females have a brood patch that is used for incubation. To assess pheomelanin-based coloration, a Nikon D3100 camera was used to photograph the breast feathers of each owl alongside a white balance card (WhiBal G7 White Balance Pocket Card) that served as a standard. We obtained a measure of the extent of reddish colour on the breast feathers of each owl (Charter et al., 2012) with the digital photography software “Barn Owl” (The Signal and Image Processing Laboratory in the Department of Electrical Engineering of the Technion). In addition to pheomelanin-based coloration, barn owls also vary in the degree of eumelanin-based coloration in the form of black spots located at the feather tips. We quantified this coloration for each individual by: (a) counting the total number of eumelanin spots within a 60×40 mm frame that was overlain on the breast of the owl, and (b) measuring the diameter of 10 spots within the frame to the nearest 0.1 mm and then calculating the mean spot diameter.

To avoid negatively affecting breeding success, all adults were returned carefully to their nest box, which featured an entrance that was blocked with a pillow that was attached to a rope. This design enabled us to remove the pillow from a distance, thereby limiting the number of owls that are flushed out of the nest box after capture (M. Charter, unpublished data).

To determine the diet of the birds, Bushnell Trophy Cam HD Aggressor No-Glow Trail Cameras were placed in the nest boxes when the oldest nestling was 30 days old and when both parents took an active role in foraging. Prey specimens were identified to the species level when possible. We used Rstudio 1.2.1335 (RStudio Team, 2018) to pair the date and time of the prey observed by the camera with the date and time that an adult owl arrived at the nest box as determined by the ATLAS tracking system (see below). This procedure allowed us to determine the diets of individual males and females, because the tracking system provided the individual identity of the forager and the cameras provided the prey type. We used data collected over a four-day period after the camera was placed in the nest box.

2.2 | Movement data

We monitored owl movement across the Hula Valley by fitting all adults with an ATLAS tracking device (Weiser et al., 2016) using a Teflon harness (total weight of the device and harness was 13 g). ATLAS wildlife tags provided the date and time at which the owls were at particular XY coordinates (Figure 1c). The localizations could

occur as frequently as once every 4 s, but there were sometimes gaps between time points if the ATLAS system could not localize an individual at a particular time point due to the signal of the tags being obscured as a result of the owl moving close to the ground, through dense trees, or into an area with weaker coverage by the ATLAS system. Movement data were analysed for 15 days following the collection of the cloacal swab, except in the case of two owls, one with only 11 days of data available and the other with only 14 days. For each night, we analysed 10 hr of data collected from 7:00 p.m. until 5:00 a.m. Across the 15 day period, the average number of observations per owl was 46,403, with a range from 3,821 to 89,763.

We used Rstudio to analyse all movement data. Owl movement was divided into two categories: at its nest, when the owl was within 40 m of its nest, and away from its nest, when the owl was >40 m from its nest and probably hunting or travelling to, or from, hunting sites. We calculated the time that each owl was at or away from its nest each night, averaged these values across the two-week period, and then computed the proportion of time that the owl was at its nest. We used the kernel estimation and the utilization distribution (Worton, 1989) to estimate the home range during the two week period using the R-package ADEHABITATHR (Calenge, 2006) with the following parameter settings: the ad hoc method for smoothing, a grid size of 2,000, a grid extent of three, and a home range at the 99th percentile level. We calculated the maximum and median displacement for each individual each night from the straight-line distances between each ATLAS localization and the owl's nest location, and then averaged these nightly values over the two week period. There was a negative correlation between the proportion of observed to expected localizations of an owl and its median displacement ($p = .04$) and home range ($p = .02$), with a similar trend for maximum displacement ($p = .11$). Thus, we generally have more localization data on owls with small home ranges than those with large home ranges, because the former were less likely to be missed as a result of passing through areas of low coverage in the ATLAS system.

2.3 | Microbial DNA extraction and sequencing

Cloacal swab samples were randomly assigned to different sets for DNA extraction. We implemented this procedure to avoid having all samples collected from a particular time, place, or category be grouped together during extraction, because of the need to control for the bacteria in DNA extraction kits (Salter et al., 2014; Weiss et al., 2014). Swabs were placed into the bead tubes with forceps that had been flame sterilized to avoid contamination. Before bead-beating, the tubes were heated to 65°C for 10 min to promote cell lysis and increase the reaction rate between the lysis buffer and the faecal material (PowerFecal DNA isolation kit: Mobio Laboratories Inc.). DNA was extracted with a Qiagen PowerLyzer PowerSoil DNA Kit. The bead-beating step was performed with a PowerLyzer homogenizer set at 3,500 rpm for 16 cycles of 30 s on and 30 s off.

DNA was quantified with a Qubit fluorometer using 5 μl of each sample. Samples were concentrated in a Centrivap vacuum

centrifuge from a volume of 95 to 40 μ l. We then sent 20 μ l to the Argonne Sequencing Center at Argonne National Laboratory, Lemont, IL, USA for PCR amplification and sequencing. The V4 region of the 16S rRNA gene was amplified using the primers 515F and 806R that also included adapter sequences for Illumina sequencing and Golay barcodes (Caporaso et al., 2012) on the forward primer. PCR reactions included 1 μ l of DNA (or more if the initial amplification failed), 9.5 μ l of MO BIO PCR Water (Certified DNA-Free), 12.5 μ l of QuantaBio's AccuStart II PCR ToughMix, and 200 pM of each primer. In the PCR, DNA was denatured at 94°C for 3 min, then cycled 35 times at 94°C for 45 s, 50°C for 60 s, and 72°C for 90 s, followed by a hold at 72°C for 10 min after cycling. Three independent PCRs were performed for each sample, which were then combined. Equimolar amounts of each sample were then pooled before sequencing on a 151 bp paired-end run of an Illumina MiSeq.

2.4 | Bioinformatics and data filtering

We demultiplexed the sequence data using QIIME2 (Bolyen et al., 2019) and followed the workflow established by Callahan, Sankaran, Fukuyama, McMurdie, and Holmes (2016) for processing the sequences in R (R Core Team, 2018). In brief, we removed the first 10 bases of each read and then inferred sequence variants using DADA2 (Callahan, McMurdie, et al., 2016). Variant inference was based on the pool of all sequence reads. After merging the forward and reverse reads, chimeric sequences were removed. We used the SILVA 132 taxonomy database (Glöckner et al., 2017; Quast et al., 2012) for taxonomic classification, with the training data obtained from <http://benjjneb.github.io/dada2/training.html>. We used the R package DECIPHER (Wright, 2015) to align sequences and the package PHANGORN (Schliep, 2010) to construct a maximum likelihood phylogeny. The phylogenetic tree, taxonomy, operational taxonomic unit (OTU) table, and metadata for the sequence data were joined together in phyloseq (McMurdie & Holmes, 2013) for analysis. The OTU table consisted of unique amplicon sequence variants (Callahan, McMurdie, & Holmes, 2017) and not groups of sequences clustered based upon a certain percentage of sequence divergence, which gave a fine-scale resolution of the bacterial diversity in the samples.

Bacteria from the DNA extraction kits, environment, or people extracting the samples serve as contaminants when characterizing an individual's microbiota (Salter et al., 2014; Weiss et al., 2014). To identify and remove such contaminants, we sequenced five negative control samples, which were processed identically to the cloacal swab samples except that a swab was not added to the tubes. We also sequenced two blank control samples, which consisted of UltraPure distilled water (Invitrogen) that went through the PCR amplification, but not the DNA extraction process. In this manner, we identified 551 contaminating sequences, which were removed from the data set. In addition to contaminants, we removed any sequences that were not assigned to the kingdom of bacteria, that could not be assigned to a phylum, or that were assigned to mitochondria or

chloroplasts. We filtered the resulting data set to only include sequences that were found in more than one individual, because rare sequence variants could easily be due to sequencing errors or rare contaminants from the environment. A total of 3,207 bacterial OTUs remained after filtering.

The average number of reads across our samples was 8,850, with a minimum of 259 and a maximum of 34,772 per individual. We excluded from the analysis six samples that had fewer than 1,000 reads and then rarefied the remaining samples to an equal sequencing depth (1,176 reads) to standardize our sequencing effort across samples (Weiss et al., 2017). We used a random number seed of 999 when rarefying the data. We excluded from the analyses one female owl that did not lay eggs and was therefore likely to be a nonbreeding adult (floater). We also excluded one male owl sample that had been collected 40 days before it had nestlings, because all other samples were collected after the nestlings had hatched. In total, we had 55 samples, of which 39 were from females and 16 were from males, all of which were actively breeding during the study period.

2.5 | Statistical tests

All statistical tests were conducted using R 3.5.1 (R Core Team, 2018). Visualization of the data was performed using functions in phyloseq and ggplot2 (Wickham, 2016). We used Fisher's exact test to compare the frequencies of prey types brought by males and females to the nest boxes.

We measured the alpha diversity of the cloacal swab samples using the Chao1 estimator of the number of species (Chao, 1984; Kim et al., 2017) with a log base 10 transformation to normalize the data. Filtering out sequence variants that were only found in a single individual (see above) could potentially affect our estimates of alpha diversity, but we found that estimates of alpha diversity with and without prevalence filtering were highly correlated ($R^2 = .832$, $p = 2.2 \times 10^{-16}$). Sample sizes for some of the phenotypic traits of the owls varied with the amount of field data available for them. The sample sizes for the traits that differed from the total possible sample size of 55 individuals are as follows: wing length (54), mass (53), clutch size (50), brood size at fledging (54), fledging success (49), and egg productivity (40). The colour phenotypes that we measured mainly showed variation across females, so we restricted our analyses to the females when testing for effects of breast coloration ($N = 38$, log-transformed), number of spots on the breast ($N = 39$), and spot diameter ($N = 39$). All movement metrics had a sample size of 46 individuals. We used a log base 10 transformation on the average median displacement per day, the average maximum displacement per day, and the home range area so that these variables would better match a normal distribution.

Some of the owl traits were correlated with one another. The age of the oldest nestling ($\chi^2_1 = 11.27$, $p = .0007$) and fledging success ($F_{1,32} = 6.67$, $p = .015$) were each negatively correlated with laying date, but the age of the oldest nestling was not correlated with fledging success ($F_{1,32} = 1.93$, $p = .174$). All of the following movement metrics

were correlated with one another: median displacement versus maximum displacement ($R^2 = .66$, $p = 5 \times 10^{-12}$), median displacement versus home range area ($R^2 = .81$, $p < 2 \times 10^{-16}$), and maximum displacement versus home range area ($R^2 = .84$, $p < 2 \times 10^{-16}$). To simplify, we reported on a subset of independent traits in the main text and reported on the other correlated traits in the Supporting Information. We analysed effects of the age of the oldest nestling in the main text, because this variable provides a direct measure of where the adult owl was in its nestling period when the cloacal swab was taken; laying data is therefore reported in the supplemental text. We reported on the average median displacement travelled per day in the main text, because this movement metric better matched a normal distribution than maximum displacement or home range area.

We used linear models as implemented in the car R-package (Fox & Weisberg, 2011) to evaluate which factors were correlated with bacterial alpha diversity. Using one-way ANOVA, we found that the sex of the bird was correlated with bacterial alpha diversity. When testing for possible correlations of each of the other variables with alpha diversity, we performed an ANCOVA that included the variable of interest, sex as a factor, and an interaction between sex and the variable of interest. If we detected a significant interaction, we implemented separate linear models for males and females in order to determine which sex had an association with changes in alpha diversity. We used type II ANCOVAs unless there was a significant interaction, in which case we used type III. With the results of the ANCOVAs, we then constructed a multivariate model of all independent variables that were correlated with alpha diversity.

We visualized differences in bacterial communities among owls (i.e., beta diversity) with a principal coordinate analysis. We calculated distances among samples using the Jaccard, Bray-Curtis, UniFrac, and weighted UniFrac metrics to determine whether patterns in our data changed with different methods of measuring differences in microbial communities. Jaccard and UniFrac are based on presence/absence of bacterial OTUs, whereas Bray-Curtis and weighted UniFrac also incorporate information on the abundance of the OTUs. UniFrac used phylogenetic information for the OTUs to calculate the distance between any two samples as the fraction of the branch-lengths of the phylogeny that were not shared between samples (Lozupone & Knight, 2005). Weighted UniFrac is a variant of UniFrac that weights the branch-lengths by the relative abundance of the OTUs in the samples (Lozupone, Hamady, Kelley, & Knight, 2007). We used the adonis function in the R-package vegan (Oksanen et al., 2018) to perform a permutational multivariate analysis of variance (PERMANOVA) to test for differences in community distances among groups with 9,999 permutations of the data. Significant differences in the PERMANOVA may result from either differences in location or dispersion, so we used the betadisper function in vegan to test for homogeneity of group dispersions. No significant differences in dispersion were detected.

We tested for differential abundance of bacteria between males and females using DESeq2 (Love, Huber, & Anders, 2014) within the phyloseq package. We first tested for differential

abundance of individual OTUs. However, differential abundance may occur at higher taxonomic levels than the OTU, if all OTUs within a particular taxonomic category are functionally equivalent to the host. Therefore, we used taxonomic agglomeration at the level of bacterial genera and families as classified by SILVA to test if higher level groupings of bacteria were differentially abundant between males and females. In all cases, we report p -values that have been adjusted for multiple testing using the Benjamini-Hochberg correction.

Studies of the microbiota of wild populations of animals are still quite new, so major factors that could affect their bacterial communities may be unknown. Many of our analyses were exploratory in nature, rather than hypothesis-driven, as we sought to capitalize on our rich data set on barn owls to identify potential factors that could be linked to their bacterial communities. These exploratory analyses meant that we tested whether the microbiota was correlated with many different phenotypes, which leads to an increased risk of false positive findings as a result of the number of statistical tests (Rice, 1989). Adjusting our p -values for the number of tests could be overly conservative (Moran, 2003), so we instead report on the results of all of our statistical tests (both significant and nonsignificant) to make the readers aware of the extent of testing and that p -values close to an $\alpha = 0.05$ should be treated with caution.

3 | RESULTS

3.1 | Alpha diversity

3.1.1 | Significant life history traits

Male owls had significantly less bacterial alpha diversity than females (Figure 2a, ANOVA, $R^2 = .079$, $F_{1,53} = 5.6$, $p = .021$). Alpha diversity was significantly related to an interaction between sex and clutch size (Figure 2b, ANCOVA, $R^2 = .169$, $F_{1,46} = 7.18$, $p = .010$), with higher alpha diversity associated with larger clutch sizes for males ($p = .01$), but not females ($p = .46$). Alpha diversity was significantly related to an interaction between sex and fledging success (Figure 2c, $R^2 = .14$, $F_{1,45} = 6.4$, $p = .015$), with females with higher fledging success tending to have more alpha diversity ($p = .09$) and males with higher fledging success tending to have less alpha diversity ($p = .10$). There was a nominally significant interaction between sex and the age of the oldest nestling ($F_{1,51} = 4.03$, $p = .050$), with female ($p = .01$), but not male ($p = .49$), alpha diversity increasing with the age of the oldest nestling at the time of sampling (Figure 2d).

3.1.2 | Nonsignificant life history traits

Body size was not a significant predictor of alpha diversity, whether measured by mass (Figure S1a, $F_{1,49} = 0.39$, $p = .54$),

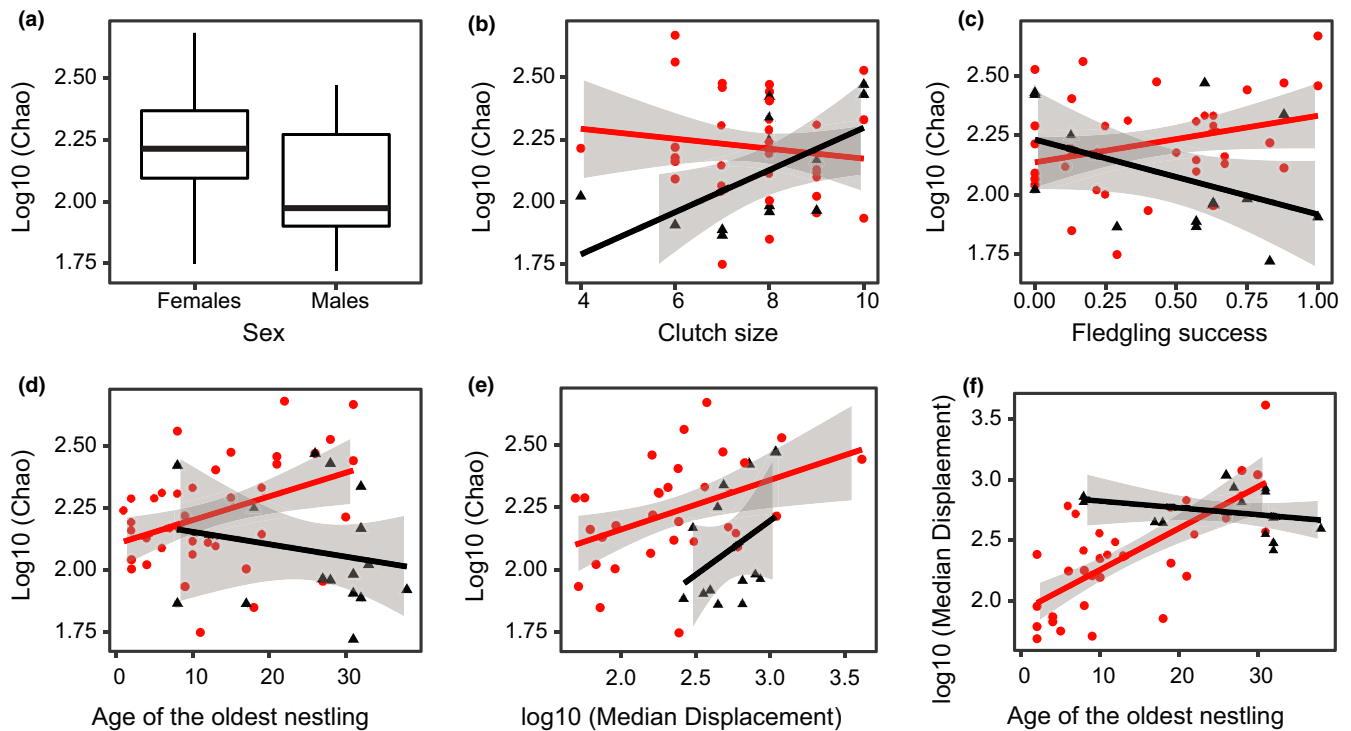


FIGURE 2 Alpha diversity related to owl phenotypes, reproduction, and movement. (a) Boxplots for males and females of the number of bacterial OTUs (i.e., alpha diversity) estimated by the Chao1 metric. (b) Alpha diversity related to clutch size, (c) fledgling success, which is the percentage of young that fledged, (d) the age of the oldest nestling, and (e) average median displacement per day from the nest in metres. (f) Relationship between the age of the oldest nestling and median displacement. Females are denoted by red points and males by black triangles. Shaded grey areas are the 95% confidence intervals around trend lines

TABLE 1 Model of uncorrelated factors that explain variation in bacterial alpha diversity as measured by the Chao1 metric ($R^2 = .35$)

Dependent variable	Independent variables	Sum sq	df	F-value	p-Value
log10 (Chao1)	Sex	0.144	1	4.08	.051
	Log10 (Median displacement)	0.207	1	5.87	.021
	Fledging success	0.002	1	0.05	.822
	Clutch size	0.174	1	4.92	.033
	Sex*Fledging success	0.128	1	3.64	.065
	Sex*Clutch size	0.158	1	4.50	.041
	Residuals	1.233	35		

wing length ($F_{1,50} = 0.29$, $p = .59$), tarsus ($F_{1,51} = 0.75$, $p = .39$), or mass divided by tarsus ($F_{1,49} = 1.2$, $p = .27$), nor were there any significant associations between alpha diversity and the interactions of these factors with sex (all p -values > 0.39). Neither brood size at fledging ($F_{1,50} = 0.12$, $p = .73$) nor an interaction of sex and brood size at fledging ($F_{1,50} = 1.39$, $p = .24$) were associated with alpha diversity. Neither egg productivity ($F_{1,36} = 1.78$, $p = .19$) nor an interaction between sex and egg productivity ($F_{1,36} = 2.41$, $p = .13$) were associated with alpha diversity. There was no association between alpha diversity and breast coloration ($F_{1,36} = 1.56$, $p = .22$), the number of spots on the breast ($F_{1,37} = 0.22$, $p = .64$), or the diameter of the spots ($F_{1,37} = 0.14$, $p = .71$) for female owls.

3.1.3 | Movement

Both median displacement ($F_{1,42} = 6.6$, $p = .014$) and sex ($F_{1,42} = 13.3$, $p = .0007$), but not their interaction ($p = .91$), were significantly related to alpha diversity in a model containing both factors ($R^2 = .206$). Individuals that traveled greater distances away from their nest had higher alpha diversity (Figure 2e). Alpha diversity was not correlated with the proportion of time spent near the nest box ($F_{1,42} = 0.001$, $p = .97$) nor with an interaction between sex and the time spent near the nest box (Figure S1b, $F_{1,42} = 1.66$, $p = .20$). Although both males and females had similar relationships between alpha diversity and the movement metrics, there were differences in movement behaviour of the two sexes. The median displacement traveled was significantly

related to an interaction between sex and the age of the oldest nestling ($F_{1,42} = 14.4$, $p = .0005$, $R^2 = .523$), with females, but not males, traveling further distances as the nestlings got older (Figure 2f).

3.1.4 | Multivariate model

We constructed a model of independent factors correlated with alpha diversity (Table 1). The age of the oldest nestling was left out of this model, given its strong correlation with median displacement from the nest (Figure 2f) and weak correlation with alpha diversity (Figure 2d). The model had an $R^2 = .35$, with a significant effect of median displacement traveled from the nest ($p = .021$), a significant interaction between sex and clutch size ($p = .041$), a nominally non-significant interaction between sex and fledging success ($p = .065$), a nearly significant main effect of sex ($p = .051$), and a nonsignificant main effect of fledging success ($p = .822$).

3.1.5 | Beta diversity

Male and female owls showed differences in the composition of their bacterial communities (Figure 3, Figure S2). The difference between the sexes was apparent across all the metrics of community difference, with the male and female samples differing on axis 2 of the principal coordinate analysis plots. We assessed the differences between male and female communities by using PERMANOVA tests, which were significant for UniFrac (Figure 3a, $p = .0016$) and Jaccard (Figure S2a, $p = .0214$) distances, nearly significant for weighted UniFrac distances (Figure 3b, $p = .0753$), and not significant for Bray-Curtis dissimilarity (Figure S2b, $p = .2034$). Individuals in the mated pairs of owls had different bacterial communities (Figure 3c), despite sharing the same nesting area.

3.2 | Differential abundance analyses

The barn owl microbiomes were dominated by the bacterial phyla Actinobacteria, Bacteroidetes, Firmicutes, and Proteobacteria (Figure 4a). Male and female samples had generally similar proportions of these phyla (Figure 4a).

The test for OTUs that were differentially abundant between males and females revealed only a single OTU with a strong bias between the sexes (log2 fold change = -4.32 , $p = .084$) that had higher abundance in females (Figure 4b). This OTU was an outlier in comparison with all other OTUs, all of which had p -values greater than 0.998. This OTU was in the genus *Lactobacillus* (family Lactobacillaceae, phylum Firmicutes). No genera were differentially abundant between males and females: *Lactobacillus* had a p -value of .520 and all other genera had p -values greater than 0.996. The test for differentially abundant families between males and females (Figure 4c) revealed that the family Actinomycetaceae was significantly more abundant in males (log2 fold change = 4.22, $p = .000009$) and the

family Lactobacillaceae was significantly more abundant in females (log2 fold change = 2.88, $p = .020$).

3.3 | Role of diet and foraging location

Males and females brought similar types and proportions of prey items to the nest (Figure 5), including voles (Günther's vole, *Microtus guentheri*), mice (house mouse, *Mus musculus*), and jirds (Tristram's jird, *Meriones tristrami*). In total, females brought 95 voles (64.6%), 14 mice (9.5%), four jirds (2.7%), and 34 unknown (23.1%) items of prey, whereas males brought 79 voles (62.7%), 11 mice (8.7%), one jird (0.8%), and 35 unknown (27.8%) items of prey. The frequencies of the different types of prey did not differ between males and females across all the individuals ($p = .60$). There were five nest boxes where we had diet data for both the male and female at the nest. In all five cases, the frequencies of prey types did not significantly differ between males and females (box 155, $p = .29$; box 157, $p = 1$; box 219, $p = .46$; box 232, $p = 1$; box 238, $p = .38$).

4 | DISCUSSION

We observed that differences in host movement patterns were correlated with differences in their microbiota, which suggests that the study of movement ecology offers a potentially valuable new way to understand host microbial communities. We do not know of any other studies that have examined the movement ecology of individual animals in relation to their microbiota, with the single exception of a laboratory study that found that locomotor behaviour in *Drosophila* is influenced by their microbiota (Schretter et al., 2018). In the owls that we studied, we found that alpha diversity was higher in individuals that moved greater distances away from the nest each day (Figure 2e). There are at least two potential explanations for this pattern. First, the association of larger movement distances with greater bacterial diversity could occur if owls that move through and forage over a larger area are colonized by a greater number of distinct species of bacteria. Dispersal of microbes can affect host microbiota (Burns et al., 2017), and host movement may effectively facilitate bacterial dispersal and colonization of the host. A study of wild populations of different mammal species suggested that bacterial types are dispersal limited at large geographic scales, because sympatric populations of host species were more similar than allopatric populations (Moeller et al., 2017). To explain the pattern we observed in owls, bacterial types would have to vary at the scale of a few kilometers or less (Figure 1c), and host alpha diversity would need to be affected by encounter rates with new bacteria. An alternative explanation for the pattern of higher bacterial diversity in individuals that move greater distances is that differences in host behaviour and physiology could lead to a correlation between alpha diversity and movement. For example, the dominance or age of owls could affect their territory sizes and movement patterns as well as physiological factors that could causally influence their microbiota such as hormone levels or their immune systems.

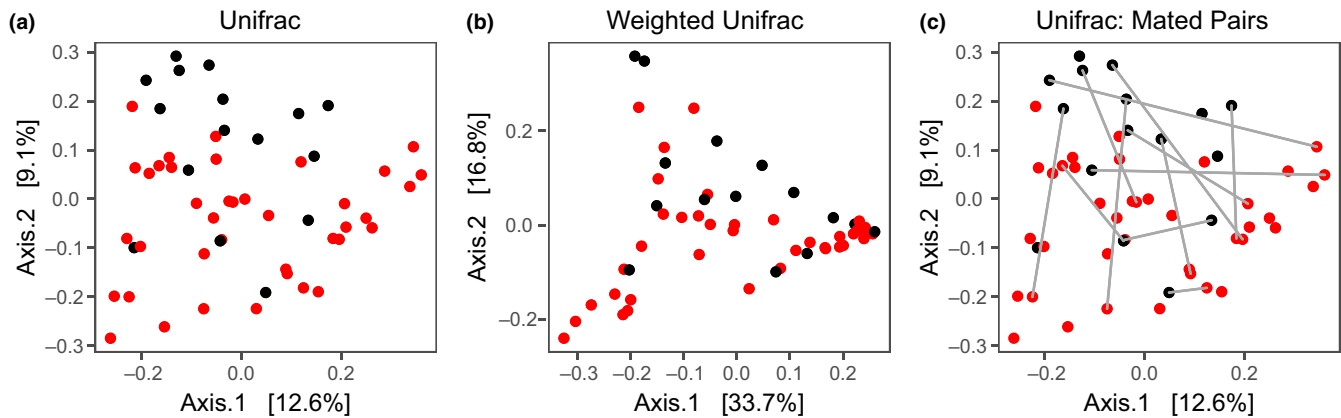


FIGURE 3 Principal coordinate analysis plots of the bacterial community composition of males (black points) and females (red points). Differences in the bacterial communities were measured by UniFrac (a & c) and Weighted UniFrac (b) distances. Grey lines in (c) connect mated pairs of owls to highlight differences between owls that shared the same nest-box. The percentage of variation explained is given for each axis

Both stress hormones (Noguera et al., 2018; Stothart et al., 2016) and sex hormones (Escallón et al., 2019; Yurkovetskiy et al., 2013) can affect the microbiome, and hormones of both types could be associated with different movement behaviours or degrees of territoriality (Canoine & Gwinner, 2002; Davies, Beck, & Sewall, 2018).

We can use our diverse data set on the owls to begin to evaluate the hypothesis that movement and microbiota are correlated as a result of host physiological differences. Male and female owls have different hormones and distinct reproductive roles during the period in which we studied them, which could potentially alter whether and how movement is correlated with the microbiota. Despite these behavioural and physiological differences, the two sexes had similar increases in their alpha diversity with increasing amounts of movement (Figure 2e), because we did not detect an interaction between median displacement and sex for predicting alpha diversity ($p = .91$). In addition, we did not find a correlation between bacterial alpha diversity and the number or the size of the eumelanin spots on the breast of the owls. Variation in these spots has previously been linked to the immune function of the owls (Roulin et al., 2000, 2001) as well as to the owls' boldness or timidity as nestlings (Peleg, Charter, Leshem, Izhaki, & Roulin, 2014). Thus, our data at hand suggests that host physiological differences may not lead to a correlation between movement and microbiota. However, more detailed studies on movement, behaviour, and physiology are needed to fully evaluate this proposition and the bacterial dispersal hypothesis.

Movement data can help explain some of the correlations we observed between parental traits and alpha diversity. We observed that alpha diversity in females, but not males, increased as their chicks aged (Figure 2d). We also observed that females, but not males, moved longer distances as their chicks aged (Figure 2f). Thus, alpha diversity is likely to increase in females as the nestlings age, because females may encounter more bacteria when foraging than when they stay at the nest. This finding demonstrates that collecting movement data can be useful for interpreting differences among individuals in their microbial communities.

We observed that the male and female barn owls in our study had differences in their cloacal microbiota. Females had more diverse microbiota than males (Figure 2a) and the two sexes differed in the composition of their microbial communities (Figure 3). Several studies of wild bird species have found little or no difference between the sexes in their microbiota. These studies include a study of New World vultures (Roggenbuck et al., 2014), a comparative study of 59 bird species (Hird et al., 2015), and a study of barn swallows (*Hirundo rustica*) in the Czech Republic (Kreisinger, Čížková, Kropáčková, & Albrecht, 2015). However, a recent paper on barn swallows in Italy found that males and females differed in microbial community composition, but not in bacterial alpha diversity (Ambrosini et al., 2019). In addition, a study of rufous-collared sparrows (*Zonotrichia capensis*) found that microbial community composition differed between the sexes in breeding males and females, but not nonbreeding birds (Escallón et al., 2019). Our study thus provides one of the first examples of sexual differences in both the composition and alpha diversity of the microbiota in a wild bird species. Differences in the microbiomes of males and females have been observed previously in model systems including humans (Mueller et al., 2006) and mice (Fransen et al., 2017; Markle et al., 2013; Yurkovetskiy et al., 2013), suggesting that perhaps finding sexual differences simply requires sufficient sample sizes and/or environmental controls to detect effects. Alternatively, sexual differences might be confined to the breeding season (Escallón et al., 2019) or may be obscured by the homogenizing effects of mating (White et al., 2010). While sexual differences in cloacal microbiota have now been observed in a few bird species, the causes of these differences are largely unknown. Our study capitalized on a rich behavioural ecology data set to explore some of the possible reasons for the differences in the microbiota of male and female owls.

There are many potential reasons why female barn owls generally have different and more diverse microbial communities than males. Microbiota in the digestive system can vary with body size (Gao et al., 2018; Reese & Dunn, 2018), and female barn owls can be more massive than males during the nesting period. However, alpha diversity was not correlated with body mass in the birds we

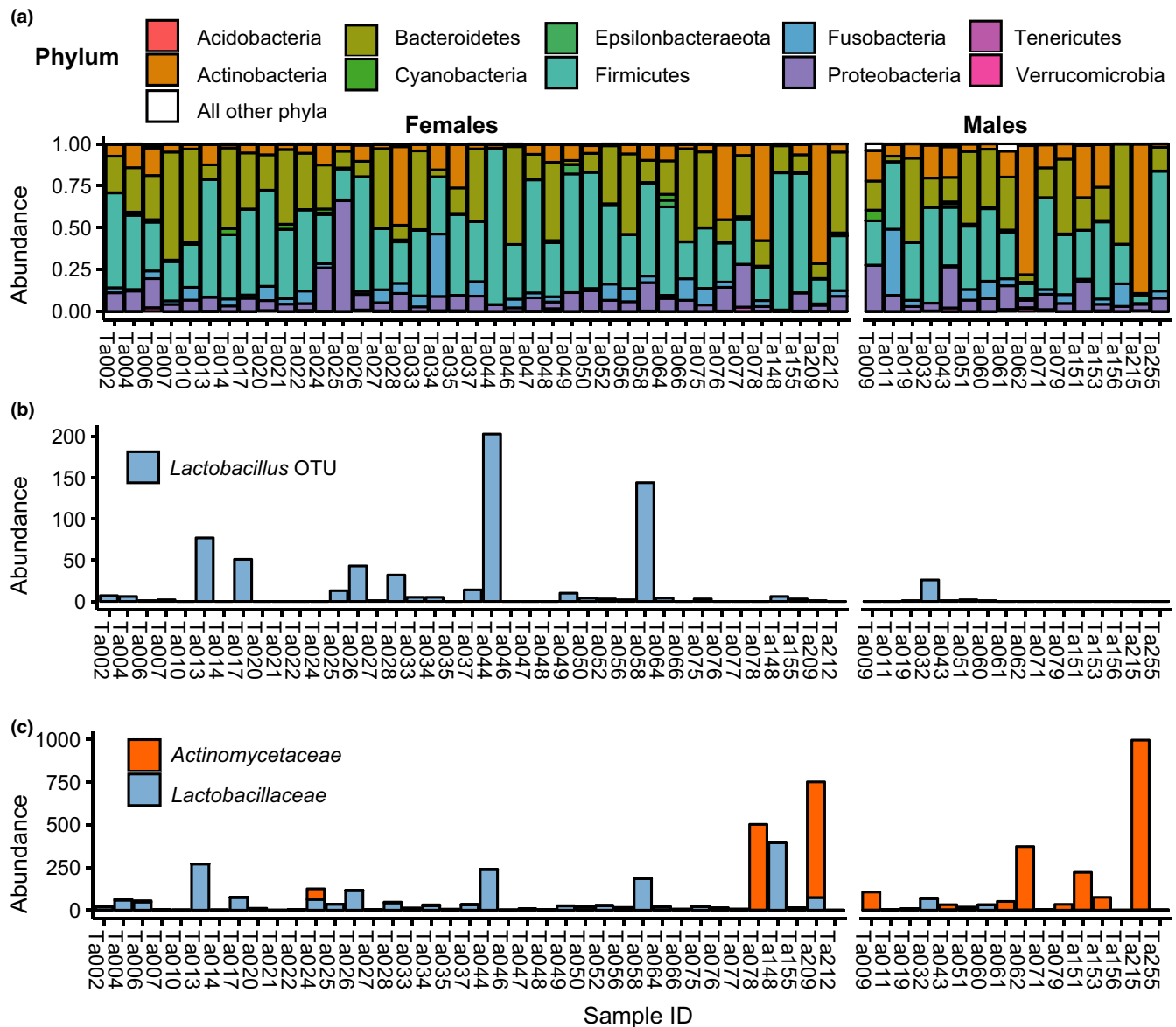
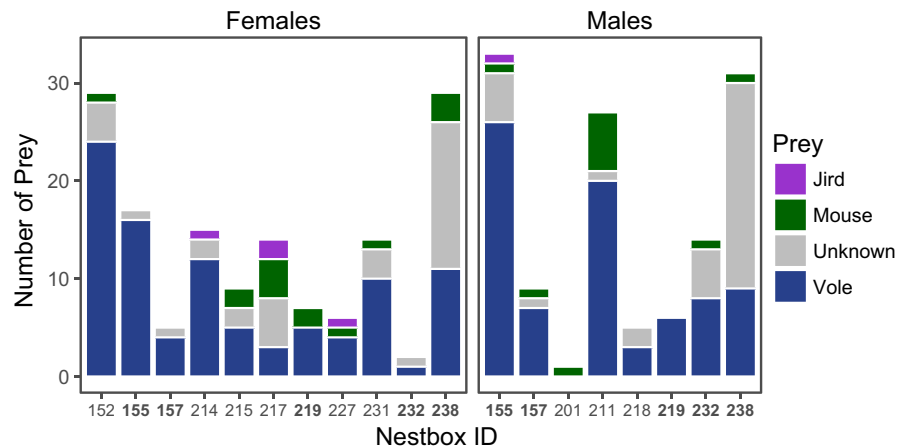


FIGURE 4 Abundance of different bacterial groups in females and males following rarefaction. (a) Proportional abundance of the top 10 most abundant bacterial phyla in each sample relative to the total abundance of all bacterial phyla (including additional phyla at low abundance). (b) Abundance of a *Lactobacillus* OTU that was differentially abundant in males and females. (c) Two families of bacteria that had significant differences in abundance between males and females. Abundance in (b) and (c) is the number of sequencing reads for each bacterial group

studied (Figure S1a), which suggests that body size is probably not responsible for the differences in microbial communities between the sexes. Another possibility is that males and females differ in foraging behaviour, which results in them having different diets and microbiomes. Work on barn owls in India showed that males and females differ in the items of prey that they bring to a nest, the mass of the prey delivered, and the number of prey deliveries (Pande & Dahanukar, 2012). While male and female barn owls specialize on different prey species in India, this does not appear to be the case in the Hula Valley in Israel, because we did not detect any differences in the types or proportions of prey delivered to nest boxes by males and females (Figure 5). Thus, the evidence at hand suggests that dietary differences are unlikely to be responsible for the

differentiation of the microbiota of male and female barn owls in Israel. Another potential reason for sex differences in the microbiota is that birds can share bacteria with their nest environment (van Veelen, Falcao Salles, & Tieleman, 2017) and female barn owls spend more time in the nestbox, which is lined with regurgitated owl pellets that probably host a lot of bacteria. We would predict that the two sexes would differ most in their microbiota early in the nesting period when the females stay in the nestbox if acquisition of bacteria from the nesting environment was responsible for the sex differences in microbiota. However, we observed the opposite pattern, with females having that greatest difference in alpha diversity when they had older nestlings (Figure 2d), which is when they resume foraging outside the nest. Similarly, neither the time spent near the nest

FIGURE 5 Number and type of prey brought to the nest box by females and males. Bold text on the x-axis denotes nest boxes that have data for both the male and female of a breeding pair



box nor an interaction between sex and the time spent near the nest box were predictive of alpha diversity. Thus, while females may acquire some bacteria from their nesting environment, this is unlikely to explain the generally higher levels of bacterial alpha diversity in females and the increase in alpha diversity when they leave the nest.

Immune system differences are another potential reason for differentiation of the microbiota between the two sexes. Males and females can differ in their immune responses (Nunn, Lindenfors, Pursall, & Rolff, 2008) and parasite loads (Zuk & McKean, 1996), which can be due to hormonal differences (McCombe & Greer, 2013; Roved, Westerdahl, & Hasselquist, 2017). Studies of mice suggest that differences in immunological responses and levels of sex hormones can lead to differences in the gut microbiota of males and females (Fransen et al., 2017; Markle et al., 2013; Yurkovetskiy et al., 2013) and, in one study, male mice had lower alpha diversity than females (Yurkovetskiy et al., 2013). Thus, our observation of generally lower alpha diversity in male barn owls could be explained by cascading effects of hormonal differences on the immune system and microbiota. Further work in barn owls is needed to directly test whether hormone levels and immune system differences can result in differentiation of their microbiota. This new area of research appears promising, given that a recent study of rufous-collared sparrows found that differences in levels of testosterone were correlated with the diversity of their microbiota (Escallón et al., 2019). Therefore, some generalizable physiological factors (e.g., testosterone levels) may exist that impact microbial diversity in both birds and mammals.

Differences in the bacterial communities of males and females were found using metrics that compared the presence/absence of species (i.e., Jaccard and UniFrac), but were less apparent with metrics that incorporated abundance information about the species (i.e., weighted UniFrac and Bray-Curtis). This finding suggests that the presence or absence of particular bacteria plays a large role in the community differences between males and females. The generally higher alpha diversities of females could mean that they are more likely to share rare OTUs, which could explain some of the similarities in their bacterial communities (Figure 3). However, differences in beta diversity between the sexes could not have been due exclusively to differences in alpha diversity, because we found that males and females had some differentially abundant bacteria (Figure 4). The

detection of sex differences in the abundance of an OTU in the genus *Lactobacillus* and the bacterial family Lactobacillaceae (which contains *Lactobacillus*) is quite intriguing, because this pattern has also been observed in other studies. A study of Japanese quail (*Coturnix japonica*) observed that females had a higher abundance of *Lactobacillus* in many regions of their gastrointestinal tract (Wilkinson et al., 2016), which is similar to our observation that an OTU in *Lactobacillus* and Lactobacillaceae were more abundant in female barn owls (Figure 4b,c). In addition, studies of mice have detected sex-specific differences for *Lactobacillus* and Lactobacillaceae as a result of differences in the immune systems of the sexes (Fransen et al., 2017; Yurkovetskiy et al., 2013). This suggests that certain bacteria may be responsive to differences in male and female environments even across such different hosts as mammals and birds. Sex differences in the bacterial family Actinomycetaceae are more difficult to explain. Increased abundance of Actinomycetaceae has been associated with obesity in humans (Peters et al., 2018). Potentially Actinomycetaceae are responding to the increased body mass that female barn owls have when they stay at the nest to guard offspring. However, we found fewer Actinomycetaceae in female barn owls, which are more massive than males when nesting, opposite to the pattern in humans. Thus, it is difficult at this time to know what factors are responsible for the differential abundance of Actinomycetaceae in barn owls.

We found that bacterial alpha diversity was correlated with two different reproductive traits: clutch size (Figure 2b) and fledging success (Figure 2c). The correlation with fledging success was not maintained in our multivariate model, which suggests that it may be a byproduct of other factors. The correlation of the bacterial alpha diversity of a male owl with the clutch size produced by his mate suggests that males may differ in behaviour, diet, or physiology in some way that influences both their own microbiota and the clutch size of their mate. For example, female barn owls rely upon their mates to provide all their prey in the days before and during the egg laying period (Taylor, 2004), so males that are better foragers might have higher cloacal bacterial diversity and also enable their female to produce more eggs.

Our research shows that the joint study of the microbiota and behavioural ecology of wild species can be fruitful for both fields of inquiry. The detailed data we collected on the behavioural ecology of barn owls showed that a subset of factors including sex, movement,

and aspects of reproduction were correlated with changes in the microbiota of the host, but that a variety of other factors (e.g., body size and coloration) were not. These findings demonstrate that detailed data on the behavioural ecology of species may be necessary to understand the diversity of factors that can alter host microbiota in wild populations and to achieve a better understanding of host/microbe interactions. While our research focused on factors that may affect the microbiota of hosts, this work also generated hypotheses about the behavioural ecology of the host species. For instance, our observation of differences between the sexes in their microbiota motivated us to test for dietary differences between males and females. Thus, studying microbiota may help inspire new ideas about the hosts' behavioural ecology. Overall, we conclude that the behavioural ecology of a host is linked to their microbiota, with sex and movement ecology being important factors that may influence the microbial community. Manipulative experiments are needed to elucidate the mechanisms that link host behavioural ecology and their microbiota. For example, movement patterns could be experimentally manipulated in aviaries or by releasing homing pigeons (*Columba livia domestica*) at varying distances from their roost. In addition, more studies of wild populations of animals are needed to test whether sex and movement ecology are generally important in structuring the microbiota of other species. We are currently studying additional bird species in the wild to determine if the links that we observed in barn owls between the microbiota and behavioural ecology of the host are generalizable to other species.

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AUTHOR CONTRIBUTIONS

R.C.K.B., W.M.G., R.N., P.L.K., and M.C. obtained funding for the study. M.C., A.C., P.L.K., R.N., W.M.G., and R.C.K.B. designed the study. S.T., and R.N. developed and maintained the ATLAS system. M.C. performed all the field data collection. G.R. analysed the movement data. S.T. helped with the microbiome sample collection. A.C. performed the laboratory work on the microbiota samples and analysed the data. A.C. wrote the paper with help from M.C., G.R., and R.C.K.B. All authors read and revised the paper.

DATA AVAILABILITY STATEMENT

The sequence data generated for the owl microbiota has been deposited in the Sequence Read Archive (BioProject ID: PRJNA578383; SRA submission: SRP226215) along with associated metadata about

the owls (Corl et al., 2019). Data collected for each individual owl and estimates of the bacterial alpha diversity for each owl can be found in Table S1.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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